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Acquisition and epidemiology of antibiotic-resistant *Escherichia coli* in a cohort of newborn calves

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Objectives: The acquisition of antibiotic-resistant commensal *Escherichia coli* was examined in a cohort of newborn calves.

Methods: Faecal samples were collected weekly from calves over a 4 month period and screened for *E. coli* resistant to ampicillin, apramycin and nalidixic acid at concentrations of 16, 8 and 8 mg/L, respectively. *E. coli* viable counts were performed on samples from a subset of calves.

Results: All calves acquired ampicillin- and nalidixic acid-resistant *E. coli*, while only 67% acquired apramycin-resistant *E. coli* during the study. Sixty-seven per cent of samples were resistant to at least one of the three antibiotics. Prevalence of ampicillin and nalidixic acid resistance was high initially and declined significantly with age ($P < 0.001$). No temporal or age-related pattern was observed in the prevalence of apramycin resistance. Housing the cohort had a significant effect on the prevalence of nalidixic acid resistance ($P < 0.001$). Total and ampicillin- and nalidixic acid-resistant *E. coli* counts declined with calf age ($P < 0.001$), with the rate of decline in ampicillin-resistant counts being greater than that for total counts ($P < 0.001$). The proportion of total *E. coli* counts that were resistant to ampicillin or nalidixic acid also declined with age ($P < 0.001$).

Conclusions: Cohort calves rapidly acquired antibiotic-resistant bacteria within days of birth. Carriage of resistant bacteria was associated with both age and housing status of the cohort.

Keywords: antibiotic resistance, *E. coli*, cattle

Introduction

Exposure to antibiotics increases the level of resistance observed in the normal commensal flora of both animals and humans.¹ Carriage of resistance by commensals has therefore been proposed as an indication of the burden of antibiotic resistance that may be present in a population,^{1,2} with low-level carriage suggested as a suitable public health goal.³ The acquisition of resistance by commensal bacteria is a serious concern, because intestinal flora act as potential reservoir pools of resistance genes which may transfer to pathogenic bacteria within the host.⁴

Current strategies to monitor the presence of antibiotic-resistant bacteria in food animals target mainly resistance in clinical specimens

and involve only periodic cross-sectional evaluations of resistance in faecal flora on a larger scale.^{2,5,6} However, such surveys do not provide any information about the dynamics of antibiotic resistance in the normal flora. When resistant bacteria are acquired during the lifetime of a food animal and whether such acquisitions are transient or not, are fundamental issues that need to be explored. In this study we therefore investigated the acquisition of antibiotic-resistant commensal *Escherichia coli* in a cohort of newborn calves.

Materials and methods

Calves ($n = 48$) born into a Scottish beef herd during autumn 2001 were enrolled in the study. Individual per-rectal faecal samples were collected

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after birth and at weekly intervals for up to 21 weeks. Calves were kept at pasture as a single group until week 10 and were then housed. Antibiotic treatment of individual animals was recorded. Sample collection was carried out in accordance with UK Government Home Office licence requirements.

Faecal samples were kept at 4°C and screened within 48 h of collection on Chromocult tryptone bile X-glucuronide agar (TBX agar; Merck) containing antibiotics (Sigma) at the following breakpoint concentrations (based on the requirement to detect the potential for resistance within a veterinary context): ampicillin, 16 mg/L; apramycin, 8 mg/L; nalidixic acid, 8 mg/L. Antibiotics were selected as examples of β -lactam, aminoglycoside and quinolone agents, antibiotic classes frequently used in farm animals. Samples were diluted 1:10 in maximum recovery diluent (MRD; Oxoid), 10 μ L spread onto non-selective (antibiotic-free) and antibiotic-containing plates and incubated overnight at 44°C. Characteristic *E. coli* colonies of a dark-blue colour were recorded, indicative of the presence of the enzyme glucuronidase.⁷ Reference strains *E. coli* NCTC 10418, NCTC 11560, JR 225 and NCTC 12900 were used to confirm the activity of antibiotic-containing plates.

For a subset of animals ($n = 24$) sufficient faeces remained to permit viable count assays. Samples were diluted 1:10 in MRD/20% glycerol and stored at -70°C. Frozen faecal suspensions (10^{-1} dilution) were defrosted within 30 min at room temperature and further dilutions (10^{-3} and 10^{-5}) prepared in MRD. Total and antibiotic-resistant counts were determined by duplicate 0.1 mL spread plates for all dilutions on unsupplemented TBX agar and agar containing the respective antibiotic at breakpoint concentration (as above). Glucuronidase-positive colonies were counted after incubation for 24 h at 44°C. Viable counts as colony forming units per g (cfu/g) were calculated as: plate count $\times 10 \times$ dilution factor.

Statistical analyses were performed using S-Plus (Insightful, Seattle, WA, USA), with $P < 0.05$ taken as significance. Changes in the cohort prevalence of resistance to the three antibiotics, and the proportion of the total counts of a sample resistant to a particular antibiotic, were analysed using generalized linear mixed-effects models with binomial errors. Calf identity was entered as a random effect to account for inherent differences between calves and the lack of independence between samples.⁸ Time, calf age and housed status were entered as fixed effects. Proportions were taken as the arithmetic mean count of an antibiotic count plate divided by that of the total count plate. Two samples where no total counts were detected were excluded; for seven samples where the ampicillin-resistant count exceeded the total count the proportion was set to 1. The freeze-thaw procedure was assumed to have an equal effect on antibiotic-resistant and -susceptible bacteria.

Changes in the total and antibiotic-resistant *E. coli* population log-transformed counts were analysed using generalized linear mixed-effects models with normal errors on data (both positive and undetected) from those calves that had at least one positive count for a particular antibiotic. Calves where no positive counts were found for a given antibiotic (ampicillin, one; apramycin, nine; nalidixic acid, four) were excluded. No qualitative differences were observed (results not shown) using positive counts only or all counts.

Results and discussion

Sixty-seven per cent of all samples ($n = 659$) carried *E. coli* resistant to at least one of the three antibiotics tested. Ampicillin-resistant *E. coli* (Amp^R*E. coli*) were detected in 64.3%, apramycin-resistant *E. coli* (Apr^R*E. coli*) in 10.3% and nalidixic acid-resistant *E. coli* (Nal^R*E. coli*) in 23.7% of samples. Both Amp^R*E. coli* and Nal^R*E. coli* were isolated from all calves at least once during the survey, whilst Apr^R*E. coli* were detected in only 67% of the cohort. There were six treatments with a fluoroquinolone agent and three with a β -lactam

agent during the study. In all cases calves had already exhibited resistance to nalidixic acid or ampicillin prior to treatment with the respective agent, or resistance was detected on the same day that the drug was administered.

The cohort prevalence of antibiotic-resistant *E. coli* changed with age and time (Figure 1). The prevalence of Amp^R*E. coli* declined linearly with age from high levels at calf birth ($P < 0.001$). Neither the decline in the prevalence of Amp^R*E. coli* with time nor changes due to housed status were significant after the effects of calf age had been taken into account ($P > 0.172$). However, there was a significant interaction between calf age and housed status ($P = 0.015$), with no change in prevalence of Amp^R*E. coli* prior to housing ($P = 0.183$), but a significant decline during the housed period ($P < 0.001$). Similarly, the prevalence of Nal^R*E. coli* significantly declined with age ($P < 0.001$), but there was no significant change with time once calf age had been taken into account ($P = 0.858$). There was a significant effect of being housed, even after taking into account age effects ($P < 0.001$): prior to housing, the prevalence of Nal^R*E. coli* remained around 50% ($P = 0.661$), but dropped at housing and remained low. The prevalence of Apr^R*E. coli* remained low with no overall age-related, temporal or housing pattern detected ($P > 0.330$).

Housing and dietary changes such as weaning may affect the prevalence of resistance by altering calf exposure to colonizing strains or changing the *E. coli* composition of the gut flora.⁹ Here it is likely that altered exposure to other animal stock and colonizing strains on the farmstead had a greater impact than diet, because cohort calves remained with their dams, unweaned, throughout the housed period. Age was a predominant factor affecting the prevalence of resistance in the cohort. However, in many animal species there is a gradual reduction in total *E. coli* count with increasing age.¹⁰ The decline in resistance prevalence could be artefactual, reflecting an overall decline in total *E. coli*, with the number of resistant bacteria falling below the detection limits of the assay, although the proportion of total *E. coli* carrying resistance may remain unchanged. Viable count analysis allowed us to examine whether the decline in the prevalence of Amp^R*E. coli* and Nal^R*E. coli* was true.

Changes in viable count profiles of total and antibiotic-resistant *E. coli* in the cohort are shown in Figure 2. Total counts declined as calves aged ($P < 0.001$). While total counts did not decline with time after age had been accounted for ($P = 0.069$), the decline in total counts with age was greater in those calves born in the early part of the study ($P = 0.005$). There was no relationship between total counts and being housed ($P > 0.078$). Amp^R*E. coli* counts also declined with age ($P < 0.001$), but were lower than total counts ($P < 0.001$), with the rate of decline greater than for total counts ($P < 0.001$). Amp^R*E. coli* counts were also lower during the housed period ($P < 0.001$), with the age-related decline in Amp^R*E. coli* counts greater during the non-housed period ($P < 0.001$). There was no change with time or interaction between time and age once the effects of housing and age had been taken into account ($P = 0.869$). The same age, time and housing status patterns were observed with Nal^R*E. coli* counts. However, there was no difference in the rate of decline in Nal^R*E. coli* counts compared with total counts ($P = 0.996$). Apr^R*E. coli* counts did not change with either age or time ($P > 0.075$).

The Amp^R*E. coli* proportion of total counts declined with calf age ($P < 0.001$), and calves born later in the study had higher Amp^R*E. coli* proportions at a given age than those born at the beginning ($P < 0.001$). Being housed had no impact on Amp^R*E. coli* proportions ($P > 0.200$). The Nal^R*E. coli* proportion declined with age ($P < 0.001$), and while there was a higher proportion of Nal^R*E. coli* in housed calves ($P = 0.019$), the Nal^R*E. coli* proportion did not change linearly with

Antibiotic-resistant *E. coli* in newborn calves

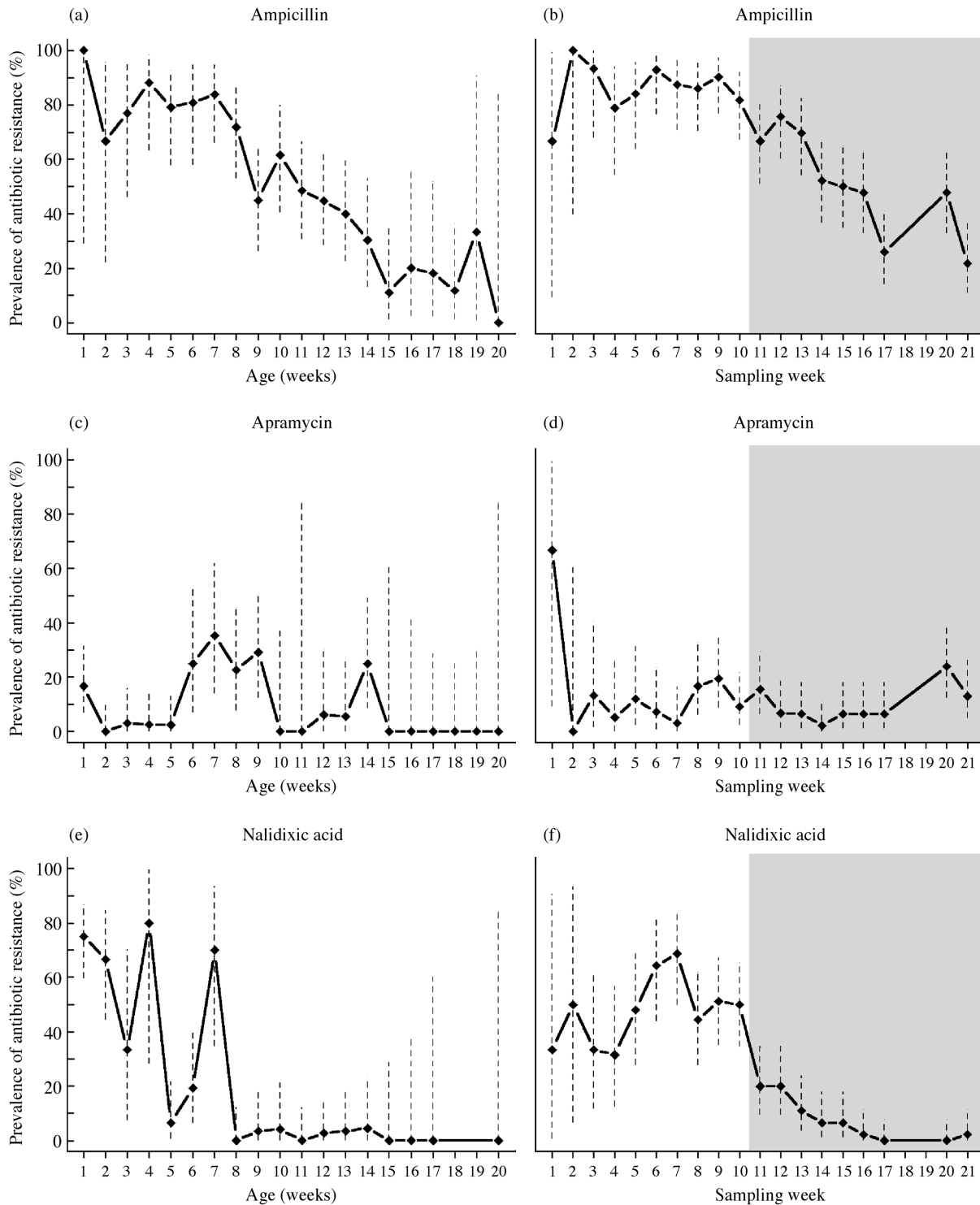


Figure 1. Prevalence of cohort calves carrying antibiotic-resistant *E. coli* as a function of calf age (a, c and e) and time (b, d and f), for Amp^R*E. coli* (a and b), Apr^R*E. coli* (c and d) and Nal^R*E. coli* (e and f). Shaded area (b, d and f) represents the period when calves were housed. Vertical dashed lines represent 95% confidence intervals for the weekly percentages.

time ($P = 0.625$). Apr^R*E. coli* proportions did not change with age or time ($P > 0.556$), but were higher during the housed period ($P < 0.001$).

Overall, viable count analysis demonstrated that while total *E. coli* counts do decline with age, the rate of decline of Amp^R*E. coli* and

Nal^R*E. coli* counts was significantly greater, with the proportion of total counts that were both ampicillin and nalidixic acid resistant also declining rapidly with age. These results indicate that cohort calves preferentially lost resistant relative to susceptible bacteria as they aged.

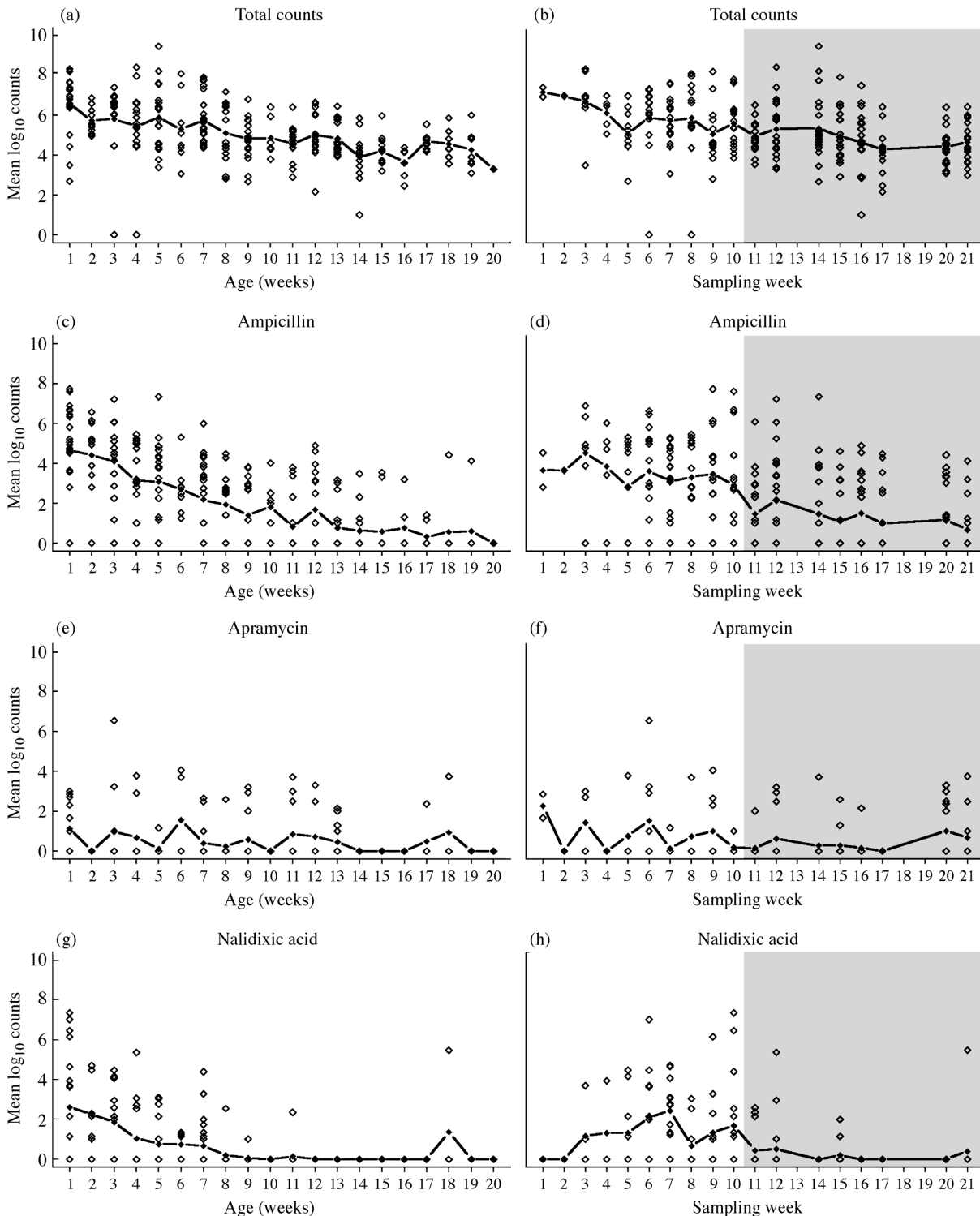


Figure 2. Change in the mean log-transformed viable *E. coli* counts of duplicate samples as a function of calf age (a, c, e and g) and time (b, d, f and h), for total counts (a and b), *Amp^RE. coli* (c and d), *Apr^RE. coli* (e and f) and *Nal^RE. coli* (g and h) counts. Shaded area (b, d, f and h) as for Figure 1. Solid lines connect the means for a given week.

In conclusion, it has been demonstrated that cohort calves were rapidly colonized by antibiotic-resistant *E. coli* shortly after birth. The prevalence of *Amp^RE. coli* and *Nal^RE. coli* within the cohort declined over the study period. Age-related changes within an animal, together with housing the cohort, had a significant negative effect upon gut carriage of resistant bacteria by cohort calves.

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Antibiotic-resistant *E. coli* in newborn calves

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